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Declaration of Dan T. Stinchcomb, Ph.D.

1. I am the Director of Biology at Ribozyme Pharmaceuticals, Inc. in Boulder Colorado. I have worked in the field of Ribozymes since 1993. My *Curriculum Vitae* is attached (Appendix A).

2. I have reviewed the specification of patent application entitled "Modified Ribozymes", US Serial No. 08/434,547 and related divisional applications–Serial Nos. 08/434,506; and 08/434,533. Following is my analysis of the efficacy of "Modified Ribozymes" *in vivo* and the potential application of ribozymes as therapeutic agents.

3. The application describes ribozymes having "catalytic" activity and "enhanced stability against chemical and enzymatic degradation." Modification of ribozymes at the sugar 2'-position with a "modifier group" such as halo, azido, sulfhydryl, amino, mono-substituted amino and disubstituted amino groups, have been shown to enhance the stability of ribozymes against nuclease degradation without affecting the ability of these modified ribozymes to cleave target RNA. One of the main purposes for enhancing the stability of ribozymes inside a cell is to improve their effectiveness as a therapeutic agent and/or as a "biocatalyst."

4. The potential for using ribozymes as therapeutic agents to diagnose and treat variety of diseases is well recognized and has been the subject of active research for the past ten years (for a review see Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023; copy enclosed as Exhibit 1).

5. While no *in vivo* data is provided in the above-captioned application, examples discussed in this declaration, and in the art, amply support the use of approaches described in the application, to successfully synthesize nuclease-stable ribozymes for sequence-specific cleavage of RNA in a cell culture or an animal model system. I believe that cell culture and animal data showing efficacy of chemically-modified ribozymes is <u>reasonably predictive</u> of their utility as a therapeutic agent.

6. Unmodified RNA is unstable in biological sera. Thus, a significant challenge in using ribozymes as drugs is to modify the RNA chemically to increase ribozyme stability while retaining ribozyme catalytic activity. A number of structural modifications have been applied to oligonucleotides in general to enhance nuclease resistance, as described in the application. The majority of this work has been carried out with hammerhead ribozymes since their small size makes them amenable to chemical synthesis and modification. Similar modifications can be readily introduced into other ribozyme motifs such as the hairpin ribozyme by adopting the teachings of the above application.

7. Chemically synthesized (and nuclease-stable) ribozymes can be directly delivered to cells and tissues using a variety of approaches known in the art (for a review see Exhibit 1). For example, ribozymes delivered by intra-articular injection have been shown to cleave target mRNA in synovial tissues (Flory *et al.* 1995, *Proc. Natl. Acad. Sci.*, 93, 754; Exhibit 2). Formulation of ribozymes in a gel form has been successfully employed to deliver ribozymes to the cornea of the eye [Ayers *et al.* 1995, *J. Controlled Rel.* (in press); Exhibit 3]. Ribozymes have also been successfully incorporated into poly L-lactic acid polymers and their delayed release in *vitro* has been documented (Lewis *et al.* 1995 *J. Cell. Biochem.* S19A, 227; Exhibit 4). There are a number of additional approaches described in the art that can be readily adopted to deliver ribozymes into a cell. The choice of delivery system can be readily made by carrying out routine and standard experiments well known in the art.

8. Following are a few examples of chemically-modified ribozyme efficacy in cell culture and animal models. These examples demonstrate the general utility of the chemically-modified ribozymes as potential therapeutic agents, as disclosed in the application.

<u>Ribozyme Efficacy In Cell Culture Model Systems:</u>

9. *Ribozymes Targeting MDR-1*: Chemically synthesized DNA/RNA chimeric ribozymes have been used to cleave *MDR-1* mRNA (Kiehntopf *et al.*, 1994, *EMBO. J.* 13, 4645; Exhibit 5). *MDR-1* encodes a phosphoglycoprotein

that can cause multiple drug resistance (MDR) in cancer cells. In this case, the hammerhead ribozyme contained DNA sequences in its binding arms and 2'-deoxy-2'-fluoro pyrimidine nucleotides in the ribozyme catalytic core and stem-loop II region. Ribozymes modified with 2'-deoxy-2'-fluoro groups are specifically described in the above-referenced applications and are known to increase nuclease resistance of the ribozymes (see also Exhibit 6). Ribozymes were delivered to cells as complexes with a cationic lipid. After treatment, drug resistant cell lines showed reduced expression of the *MDR-1* gene product, reduced ability to export rhodamine and increased sensitivity to the anti-neoplastic drug, vindesine. Interestingly, the DNA/RNA chimeric ribozymes showed enhanced efficacy relative to an all RNA ribozyme or an antisense phosphorothioate DNA control; an inactive all RNA ribozyme had no effect on gene expression or drug resistance. Synthetic chemically-modified ribozymes directed against *MDR-1* may provide a means of reversing drug resistance that can be observed in cancer patients during chemotherapy.

I performed or had performed on my behalf, the experiments discussed in Sections 10-13, *infra*.

10. *Ribozymes Targeting c-myb:* Coronary angioplasty is widely used to surgically treat atherosclerosis. Unfortunately, 35-45% of angioplasty patients develop restenosis or reocclusion of the treated vessel within 6 months of the operation. Restenosis after angioplasty is associated with activation and proliferation of underlying smooth muscle cells. The hyperproliferation and excessive matrix deposition by these cells contribute to a reduction in the diameter of the lumen and eventual occlusion of the vessel. Ribozymes capable of inhibiting smooth muscle cell activation and proliferation could be used therapeutically to reduce intimal thickening and restenosis.

The proto-oncogene, c-*myb* is thought to be a critical regulator of smooth muscle cell proliferation. Ribozymes with optimal activity were synthesized using the nuclease-stable ribozyme motif described in the application and by Beigelman *et al.,* 1995 *J. Biol. Chem.* 270, 25702; Exhibit 6). The hammerhead ribozymes contained either 1) 2'-*C*-allyl substitution at U_4 position, five ribose residues, four phosphorothioate linkages between

nucleotides in the 5′-binding arm, an inverted 3′-3′-linked nucleotide at the 3′-terminus, and 2'-*O*-methyl substitutions at all the other positions; 2) or 2′-deoxy-2'-amino substitution at U4 and U7 positions, five ribose residues, four phosphorothioate linkages between nucleotides in the 5′-binding arm, an inverted 3′-3′-linked nucleotide at the 3′-terminus and 2'-*O*-methyl substitutions at all the other positions. Ribozymes modified with 2'-deoxy-2'-amino groups are specifically described in the above-referenced applications and are known to increase nuclease resistance of the ribozymes (see also Exhibit 6).

The nuclease-stable ribozymes were applied to serum-starved smooth muscle cells as complexes with the cationic lipid, DOSPA. Active ribozymes effectively blocked serum-stimulated cell proliferation [Figures 2-6 of Jarvis *et al.,* 1996 *RNA* (in press), Exhibit 7; and Figures 3-6 of Jarvis *et al.,* 1996 (submitted for publication); Exhibit 11]. Inactive ribozymes or active ribozymes with scrambled binding arm sequences had little effect on cell proliferation. Ribozymes without modifications that render them stable to nucleases failed to dramatically inhibit cell proliferation. Thus, optimal inhibition of proliferation was sequence-specific and required a nuclease-stable catalytic core capable of cleaving the target mRNA. Indeed, when c-*myb* mRNA levels were measured by a quantitative polymerase chain reaction technique, the active ribozyme significantly reduced cellular levels of its target mRNA. The ability of these synthetic, nuclease-resistant ribozymes to inhibit smooth muscle cell proliferation raises the possibility of locally delivering ribozymes to vessel walls immediately following angioplasty procedures. Prevention of smooth muscle cell activation and proliferation by such ribozymes directed against c-*myb* may reduce the incidence of restenosis that occurs after coronary angioplasty.

11. *Ribozymes Targeting HIV-1 RNA:* Ribozymes with optimal activity were synthesized using the nuclease-stable ribozyme motif described in the application and by Beigelman *et al.,* 1995 *J. Biol. Chem.* 270, 25702; Exhibit 6). The hammerhead ribozymes contained the 2′-deoxy-2'-amino substitution at U4 and U7 positions, and an inverted 3′-3′ nucleotide at the 3′-terminus. Ribozymes modified with 2'-deoxy-2'-amino groups are specifically described in the above-referenced applications.

The nuclease-stable ribozymes, targeted against human immunodeficiency virus (HIV) LTR, were applied to $CD4^+$ 293 cells as complexes with calcium phosphate. The cells were then infected with HIV-1 and the level of viral protein, p24, production was measured at 6 days post infection. As shown in Exhibit 8, active 568 hammerhead ribozymes (Active 568 HH) effectively decreased HIV-1 p24 protein production at two different ribozyme concentrations of 3 and 6 µg. A decrease in HIV-1 p24 protein production is indicative of inhibition of HIV-1 replication. Inactive ribozymes (Inactive 568 HH), as expected, had little effect on p24 production since they are not capable of cleaving the target RNA. Thus, inhibition of viral replication was sequence-specific and required a nuclease-stable catalytic core capable of cleaving the target mRNA. The ability of these synthetic, nuclease-resistant ribozymes to inhibit HIV replication indicates the possibility of using ribozymes as potential anti-viral agents.

<u>Ribozyme Efficacy in Animal Model Systems:</u>

12. *Ribozyme efficacy in the Rabbit Knee model:* Osteoarthritis is a debilitating disease in which loss of cartilage can cause severe pain and incapacitation. The degradation of cartilage in osteoarthritic patients is correlated with excessive production of the matrix metalloproteinase, stromelysin. Nuclease-resistant ribozymes have been shown to reduce their stromelysin mRNA when injected into the synovium of rabbit knees (Flory *et al.* 1995; Exhibit 2).

The hammerhead ribozymes used in this study contained 1) 2´-*C*-allyl substitution at U_4 position, five ribose residues, four phosphorothioate linkages between nucleotides in the 5´-binding arm, an inverted 3´-3´-linked nucleotide at the 3´-terminus and 2'-*O*-methyl substitutions at all the other positions; 2) 2´-deoxy-2'-amino substitution at U_4 and U_7 positions, five ribose residues, four phosphorothioate linkages between nucleotides in the 5´-binding arm, an inverted 3´-3´-linked nucleotide at the 3´-terminus and 2'-*O*-methyl substitutions at all the other positions; 3) 2´-*C*-allyl substitution at U_4 position, five ribose residues, an inverted 3´-3´-linked nucleotide at the 3´-terminus and 2'-*O*-methyl substitutions at all the other positions; or 4) 2´-deoxy-2'-amino substitution at U_4 and U_7 positions, five ribose residues, an

inverted 3′-3′-linked nucleotide at the 3′-terminus and 2'-*O*-methyl substitutions at all the other positions. Ribozymes modified with 2'-deoxy-2'-amino groups and phosphorothioate substitutions are specifically described in the above-referenced applications and are known to increase nuclease resistance of the ribozymes (see also Exhibit 6).

Nuclease-stable ribozymes directed against the mRNA encoding stromelysin were injected in physiological saline solution and were shown to accumulate intact in synovial tissue. Twenty four hours after ribozyme administration, IL-1α was injected into the joint to induce stromelysin mRNA expression. Tissues were harvested 6 hours after IL-1α induction, synovial RNA was extracted and stromelysin mRNA was quantified by Northern or RNase protection analysis. Intra-articular injection of active ribozymes significantly reduced synovial levels of stromelysin mRNA (Figures 4-6 of Exhibit 2). Inactive or irrelevant ribozymes had no effect. Active ribozymes with several different chemical modifications (including the 2'-deoxy-2'-amino modifications described in the application), to provide nuclease-resistance, and targeting several different ribozyme cleavage sites in stromelysin mRNA were efficacious *in vivo.* Thus, nuclease-resistant ribozymes can specifically cleave target mRNAs *in vivo.* These experiments demonstrate the feasibility of using small, chemically synthesized ribozymes to impact gene expression in animal models of human disease. Furthermore, these ribozymes may be useful in reducing the active joint degradation that occurs in patients with osteoarthritis.

13. *Ribozyme efficacy in rat corneal model:* Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells and plays a role in several pathological conditions such as proliferative retinopathy and solid tumor growth. VEGF stimulates vascular endothelial cell growth and proliferation of new blood vessels *via* two transmembrane receptors, *Flt-1* and KDR.

As a novel approach to the regulation of abnormal angiogenesis, we have designed and tested ribozymes that are targeted to *Flt-1* and *KDR* mRNAs. Reduction of VEGF receptor mRNAs by ribozyme cleavage should lead to a reduced level of receptor protein and a subsequent decrease in

disease-related angiogenesis. All ribozymes used in these studies were prepared synthetically and are chemically stabilized with "modifier" groups, such as 2'-*C*-allyl and 2'-deoxy-2'-amino (specifically described in the above-referenced applications), to protect them from degradation.

We have identified a number of ribozymes targeted to both receptor mRNAs that are capable of significantly reducing VEGF-stimulated growth of human microvascular endothelial cells in culture. For example, as shown in Exhibit 12, hammerhead ribozymes targeted against two sites (site 1358 and 4229) within the *Flt-1* mRNA were synthesized with chemical modifications and tested in a cell-proliferation assay. The hammerhead ribozymes contained either 1) 2′-*C*-allyl substitution at U_4 position, five ribose residues, four phosphorothioate linkages between nucleotides in the 5′-binding arm, an inverted 3′-3′-linked nucleotide at the 3′-terminus and 2'-*O*-methyl substitutions at all the other positions (2'-*C*-allyl ribozymes); 2) or 2′-deoxy-2'-amino substitution at U_4 and U_7 positions, five ribose residues, four phosphorothioate linkages between nucleotides in the 5′-binding arm, an inverted 3′-3′-linked nucleotide at the 3′-terminus and 2'-*O*-methyl substitutions at all the other positions (2'-Amino ribozymes). Ribozymes modified with 2'-deoxy-2'-amino groups are specifically described in the above-referenced applications and are known to increase nuclease resistance of the ribozymes (see also Exhibit 6). Nuclease-stable hammerhead ribozymes targeted to both sites within *Flt-1* RNA were able to significantly inhibit VEGF-induced proliferation of human microvascular endothelial cells. The specificity of ribozyme action was demonstrated in that Fibroblast Growth Factor (FGF)-stimulated cell growth was completely unaffected by treatment with ribozymes targeting *Flt-1* VEGF receptor mRNAs.

Greater than 90% inhibition of VEGF-induced cell proliferation was observed when ribozymes directed against both *Flt-1* and KDR receptors were tested in combination. Control ribozymes, either catalytically inactive ribozymes or active ribozymes targeted to unrelated RNAs, had little or no effect on VEGF-stimulated cell growth.

Studies using a rat corneal model of angiogenesis in which VEGF protein and the chemically-stabilized ribozymes were co-delivered on a

nitrocellulose filter disk surgically implanted into the cornea have been carried out. Fluorescently-labeled ribozyme accumulated in the limbus region in or near the microvascular endothelial cells of the pericorneal vessels from which corneal neovascularization occurs. A significant portion of ^{32}P-labeled ribozyme remained intact in the eye for at least 72 hours. These two observations indicate that ribozymes accumulate and persist in the target tissue when administered in this manner.

In further studies using the same VEGF/ribozyme administration method described above, a synthetic chemically-stabilized hammerhead ribozyme (the ribozyme contained 2′-*C*-allyl substitution at U_4 position, five ribose residues, four phosphorothioate linkages between nucleotides in the 5′-binding arm, an inverted 3′-3′-linked nucleotide at the 3′-terminus and 2'-*O*-methyl substitutions at all the other positions) targeting site 4229 within *Flt-1* mRNA (Active 4229 HH) inhibited VEGF-induced angiogenesis by greater than 50% relative to corneas treated with either VEGF alone or VEGF and a catalytically inactive ribozyme (Inactive 4229 HH; Exhibit 9). These results indicate that ribozyme catalysis is required for anti-angiogenic activity. Eyes treated with ribozyme alone (*i.e.* no VEGF) showed no angiogenic response (Exhibit 9). This work suggests a therapeutic role for ribozymes in diseases characterized by VEGF-related neovascularization.

14. *Ribozyme efficacy in newborn mice:* Amelogenin (AMEL) is a highly conserved group of protein necessary for mammalian enamel "biomineralization". Lyngstadaas *et al.*, 1995, *EMBO. J.* 14, 5224 (Exhibit ~~9~~ 10), have recently shown that a locally administered chemically-modified hammerhead ribozyme (the ribozymes were substituted with 2'-*O*-allyl groups to enhance nuclease resistance) is capable of cleaving amelogenin RNA in newborn mice resulting in "a prolonged and specific arrest of amelogenin synthesis" and a subsequent inhibition of normal mineralized enamel formation (Figures 3-4 of Exhibit 10). The authors state in the abstract on page 5224 that: "[T]hese results demonstrate that synthesized ribozymes can be highly effective in achieving both timed and localized 'knock out' of important gene products *in vivo*, and suggest new possibilities for suppression of gene expression for research and therapeutic purposes."

15. The examples discussed in this declaration provide ample support for the general utility and enablement of using the approaches, described and claimed in the application, to successfully synthesize chemically-modified ribozyme that are nuclease stable and catalytically active. These ribozymes are capable of specifically cleaving target RNA in a variety of cell culture and animal model systems As described in the application, it is now possible to design *trans*-cleaving ribozymes, synthesize and stabilize them chemically, deliver them to cells, and test them for efficacy in an ever increasing number of cell and animal models. The efficay of ribozymes in cell culture and animal models are reasonably predictive of the utility of ribozymes as therapeutic agents. These results strongly suggest that the therapeutic benefits of synthetic chemically-modified ribozymes in humans will most certainly be realized.

I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: April 30 1996

Dan T. Stinchcomb

Dan T. Stinchcomb, Ph.D.